Reliability of photographic and molecular techniques for sexing northern bottlenose whales (*Hyperoodon ampullatus*)

S. Gowans, M.L. Dalebout, S.K. Hooker, and H. Whitehead

Abstract: Identifying the sex of living cetaceans can be difficult, even when species are sexually dimorphic. We compare two methods of molecular sexing (*ZFY* (zinc finger protein gene) and *SRY* (sex-determining region Y gene)) and evaluate the effectiveness of photographic techniques for identifying sex in northern bottlenose whales, *Hyperoodon ampullatus*, in the Gully, off Nova Scotia, Canada. Samples from individuals of known sex from historic Norwegian whaling (n = 19) and from recent strandings (n = 3) were used to test the reliability of the molecular techniques. Although both methods gave accurate results, the *ZFY* method was found to be unsuitable for degraded (historic) samples, owing to the large size of the target DNA fragment. Results from the two molecular-sexing methods were in agreement for biopsy samples taken from bottlenose whales in the Gully (7 males and 13 females). Photographs of the melon profile were used to assign free-swimming animals to the categories female – immature male, subadult male, and mature male. Melon photographs of adult-sized animals taken up to 7 years apart were consistently assigned to the same category. Overall, sex identification from melon photographs was in agreement with results from molecular sexing. However, animals in the category female – immature male were difficult to assign on the basis of morphological features alone.

Résumé : La reconnaissance du sexe chez les cétacés peut être difficile, même lorsque les espèces font preuve de dimorphisme sexuel. Nous comparons ici deux méthodes moléculaires d'identification du sexe (*ZFY* (gène sur l'Y codant pour les protéines à doigt de zinc) et *SRY* (le gène sur l'Y de la région déterminante du sexe)) et nous évaluons l'efficacité des techniques photographiques d'identification du sexe chez des Baleines à bec communes, *Hyperoodon ampullatus*, dans la tranchée Gully au large de la Nouvelle-Écosse, Canada. Des échantillons d'individus de sexe connu du temps des chasses des baleiniers norvégiens (n = 19) et des individus échoués récemment (n = 3) ont servi à tester la fiabilité des techniques moléculaires. Bien que les deux méthodes aient donné des résultats fiables, la méthode *ZFY* s'est avérée inadéquate dans le cas d'échantillons dégradés (anciens) à cause de la grande taille du fragment cible d'ADN. Les deux techniques moléculaires ont donné des résultats semblables dans le cas d'échantillons de biopsies recueillies sur des Baleines à bec communes de la tranchée (7 mâles et 13 femelles). Des profils du melon ont servi à assigner des individus nageant librement aux catégories suivantes: femelle – mâle immature, mâle sub-adulte et mâle à maturité. Les photographies du melon d'animaux de taille adulte prises à des intervalles pouvant atteindre 7 ans étaient toujours assignés à la même catégorie. Dans l'ensemble, l'identification du sexe sur des photos du melon sont en accord avec les résultats de la détermination du sexe par une méthode moléculaire. Cependant, les animaux de la catégorie femelle – mâle immature étaient difficiles à classifier sur la base de structures morphologiques seulement.

[Traduit par la Rédaction]

Introduction

Identifying the sex of free-ranging cetaceans is often difficult, even for sexually dimorphic species. Nonetheless, such information is of fundamental importance in studies of population structure, social organization, distribution, or behaviour. Most authors have inferred individuals to be female if they are observed near calves (e.g., Baker et al. 1987; Clapham and Mayo 1987; Slooten et al. 1993; Knowlton et al. 1994). Such inferences are troublesome for some species where "babysitting" (serial accompaniment of calves by alloparents) might occur (e.g., sperm whales (*Physeter macrocephalus*); Whitehead 1996).

Northern bottlenose whales (*Hyperoodon ampullatus*) are sexually dimorphic. They can be grossly described from their melon profile as female – immature male, subadult male, or mature male (Gray 1882). Mature males are, on average, 1 m longer than mature females (Christensen 1973) and have a flattened white melon profile. In comparison, females have a gray bulbous melon profile. Subadult males are intermediate for this character (Gray 1882; Fig. 1).

Sex may be determined from tissue samples using molecular techniques and can be used to confirm inferences from morphological features. Both *ZFY* (zinc finger protein gene) and *SRY* (sex-determining region Y gene) techniques have

Received October 5, 1999. Accepted March 16, 2000.

S. Gowans,[1] S.K. Hooker,[2] and H. Whitehead. Department of Biology, Dalhousie University, Halifax, NS B3H 4J1, Canada.
M.L. Dalebout. School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1000, New Zealand.

[1]Author to whom all correspondence should be addressed (e-mail: sgowans@is2.dal.ca).
[2]Current address: British Antarctic Survey, Madingley Road, Cambridge, CB3 OET, U.K.

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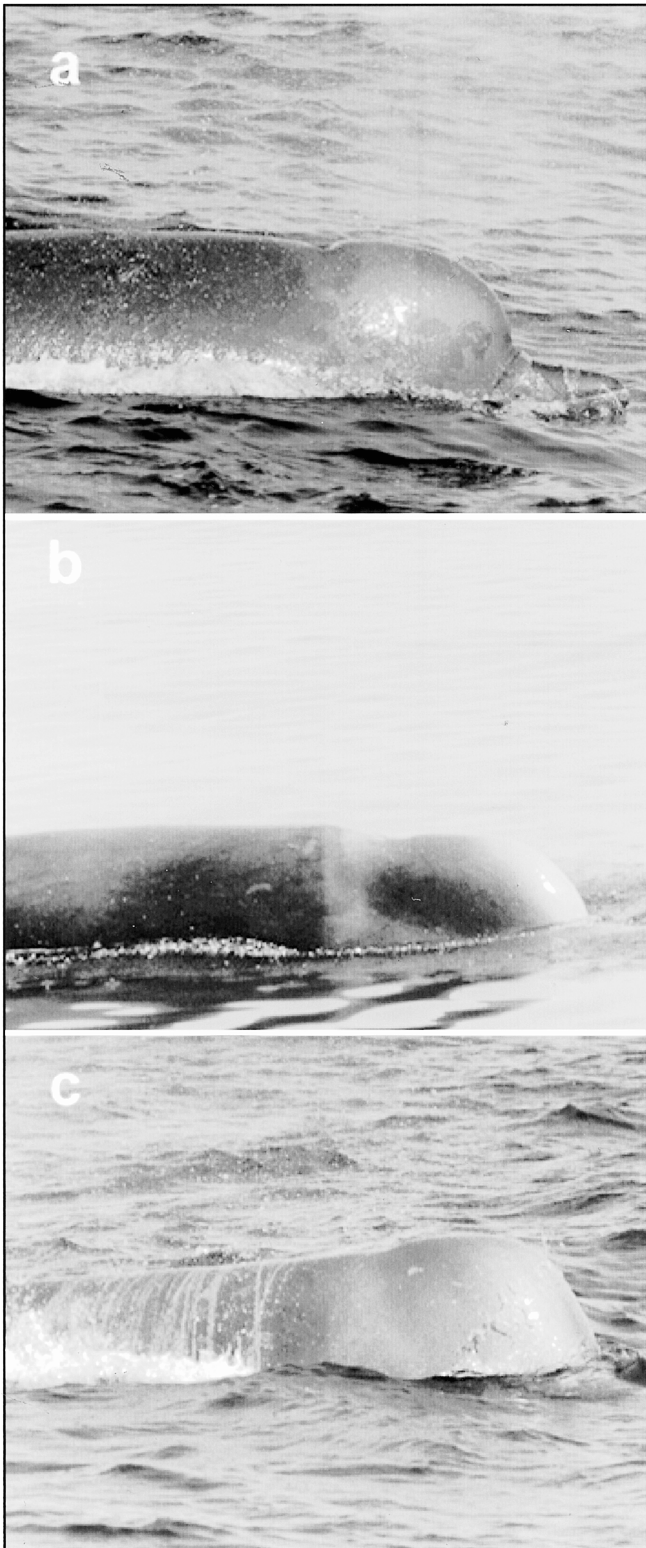
Fig. 1. Sexual dimorphism in melon shape in northern bottlenose whales. (*a*) Female – immature male. (*b*) Subadult male. (*c*) Mature male.



been used to identify the sex of cetaceans (e.g., Palsbøll et al. 1992; Patenaude et al. 1998; Baker et al. 1999) but these methods have not been used previously on beaked whales (family Ziphiidae). Tissue samples from individuals of known sex from recent strandings and from historic Norwegian whaling were used to asses the reliability of these molecular techniques for identifying the sex of northern bottlenose whales.

The sex of free-swimming northern bottlenose whales was determined using molecular assays of skin-biopsy samples. These results were used to assess the accuracy and reliability of melon-profile photographs for sex identification in this species.

Materials and methods

Molecular data

We conducted field studies from 1988 through 1997 in the Gully, off Nova Scotia, Canada (44°N, 59°W), using a sailboat (Whitehead et al. 1997). We obtained skin samples from 20 live animals using standard-biopsy techniques. All samples were taken from the flank area near the dorsal fin (for more details see Hooker 1999). A small subsample of the skin was stored in salt-saturated dimethyl sulphoxide (DMSO) solution immediately after collection. Of the 20 animals biopsied, 10 were also photographed for individual and sex identification.

Samples from animals of known sex from recent strandings (3 males) and from the historic Norwegian whaling fishery for this species in Davis Strait (12 males and 7 females) were used to test the reliability of molecular-sexing techniques. Stranding samples were provided by the Nova Scotia Stranding Network, Groupe de recherche et education sur le milieu marine, and the Department of Fisheries and Oceans Canada, Mont Joli, Quebec. Historic whaling samples were provided by I. Christensen, Institute of Marine Research, Norway. Samples from stranded animals consisted of skin and blubber preserved in salt-saturated DMSO. For the historic whaling samples, remnant gum tissue was scraped from teeth that had been collected from these animals and stored unpreserved at room temperature for 30 years prior to this study.

We determined the reliability of the *ZFY* method of Palsbøll et al. (1992) and the *SRY* method of Richard et al. (1994) for northern bottlenose whales using samples from animals of known sex. The sex of the 20 free-swimming animals sampled by biopsy in the Gully was then identified using both methods.

DNA was extracted from all samples using a modification of the Chelex-100 method (Walsh et al. 1991; N. Gemmell, unpublished), which allows longer-term storage of the DNA material obtained. The homologous *ZFY–ZFX* regions of the sex chromosomes (each ca. 1100 base pairs (bp) in length) were amplified using the polymerase chain reaction (PCR) and subsequently digested with the *Taq*I restriction enzyme (Palsbøll et al. 1992). The *SRY* locus is Y chromosome specific in most mammals (Sinclair et al. 1990), so only males will yield a PCR product in any reaction attempting to amplify a fragment of this gene. The primers SRYR1 and SRYR2, which were originally designed for sperm whales, were used to amplify a 147-bp fragment of the *SRY* locus (Richard et al. 1994). As PCR failure with this method could be misinterpreted as a false negative (female), an additional fragment of the mtDNA control region (size 500 bp) was co-amplified in the reaction, using the primers t-Pro whale and Dlp5 (Dalebout et al. 1998), to control for this. To account for the possibility that a sample might allow amplification of mitochondrial loci but not of nuclear loci, owing to DNA degradation (a common problem when working with historic material) or PCR inhibition, we also attempted to amplify a small fragment of the nuclear actin intron (223 bp) for each sample in a separate reaction. Only samples from which the actin fragment was successfully amplified were used in our final analyses.

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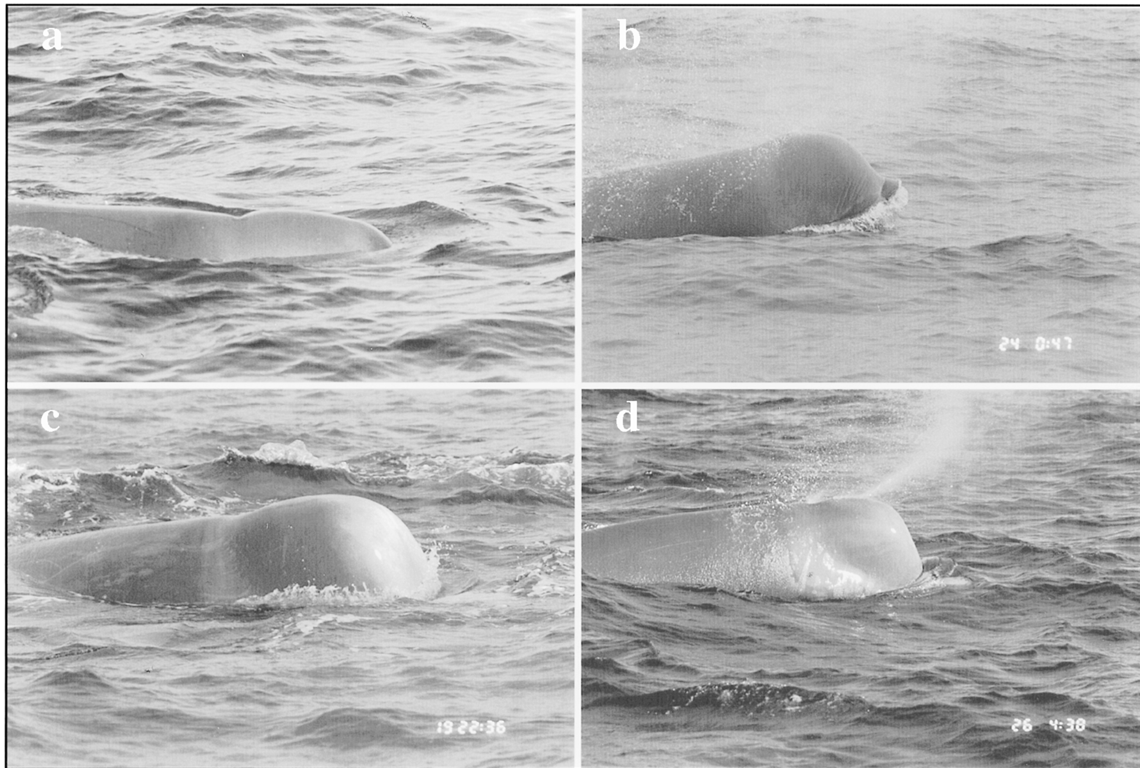
Fig. 2. Examples of various qualities of melon photographs. Photographs *a–d* illustrate melon photograph quality categories 1 (*a*, unusable) through 4 (*d*, excellent).



Photographic data

When field conditions permitted, bottlenose whales were approached to within approximately 15 m and melon and dorsal fin photographs were taken. We used Canon AT1 and ElanIIE cameras with 300-mm lenses and Ilford HP5 400 ASA black and white film for most photographs. Whenever possible, a suite of melon and dorsal photographs was taken of the same individual. Melon photographs were used to determine sex, while photographs of the dorsal fin were used for individual identification (for more details see Gowans 1999).

We viewed negatives of the melon profiles on a light table with a 10× magnifying loupe. We identified each photograph as either unknown, female – immature male, subadult male, or mature male based on figures from Gray (1882) (Fig.1). Photographs were also assigned a melon-photograph quality (MQ), which reflected the potential of the photograph to classify the individual (1, unusable; 4, excellent; Fig. 2). MQ was based on the exposure and focus of the photograph, the angle of the melon, and the amount of melon visible above the surface of the water (cf. Arnbom 1987; Gowans 1999). Melon photographs that were taken in the same suite as an identifiable dorsal fin photograph were assigned the identification number of that individual.

To assess whether melon photographs consistently and reliably allowed the age–sex of an individual to be categorized over time, sequential photographs taken of the same individual and the age–sex category to which these photographs were assigned were compared. All consecutive usable melon photographs (MQ $\geq$ 2) of the same individual were compared. As the melon profile in males changes over time, these sequential photographs were also examined for any changes in melon shape. Misclassification of individuals based on melon photographs was investigated by comparing all sets of three sequential melon photographs taken of the same individual on 3 separate days in which the categorization in the first and last photograph were the same. Sequences in which the middle photograph was classified differently from the earlier and later photographs were considered misclassifications. Three photographs were needed to differentiate between a miscategorization and the natural progression in melon shape as males matured.

Results

Molecular sexing

Amplification of the *ZFY–ZFX* locus generated a product of expected size (ca. 1100 bp) and, in all cases where PCR was successful, digestion with *Taq*I produced a sex-specific pattern similar to that reported by Palsbøll et al. (1992). However, we were unable to amplify this locus from any of the historic whaling samples. The results for the stranding samples were in agreement with their sex as determined in the field (3 males). *ZFY*-sexing was successful for all biopsy samples (7 males and 13 females; Table 1).

For the *SRY* method, preliminary amplification of the actin intron fragment to evaluate the ability of samples to amplify nuclear loci was successful for 19 of the 22 whales of known sex. In subsequent co-amplification of the *SRY* fragment with the mtDNA control region fragment, 18 of the 19 samples gave results concordant with the field records (12 males and 6 females; Table 1). Males gave a product for both the *SRY* fragment (147 bp) and the mtDNA fragment (500 bp), while females gave a product only for the mtDNA fragment. The one sample for which results were not concordant was sexed as a female using the *SRY* method, but was identified as a male in the whaling records. Attempts to co-amplify a nuclear fragment similar in size to the mtDNA fragment (500 bp) with the *SRY* method were unsuccessful. The test actin fragment was successfully amplified from all biopsy

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Table 1. Comparison of sex identification based on field observations and molecular techniques.

Field identification	Molecular identification (*SRY* and *ZFY*) Male	Female	Notes
Whaling samples			
Male (n = 12)	9[a]	1[a]	No *SRY* results for 2 samples
Female (n = 7)	0[a]	6[a]	No *SRY* results for 1 sample
Stranding samples			
Male (n = 3)	3	0	
Biopsy samples			
Mature male (n = 2)	2	0	
Female – immature male (n = 8)	1[b]	7	
Melon sex unavailable (n = 10)	6	4	

[a]Samples too degraded for *ZFY* analysis. The results of the *SRY* analysis only are presented.
[b]Individual No. 143.

Table 2. Consistency of the melon-photograph technique for sexing individuals (number of comparisons is shown).

Initial sexing	Time frame of comparisons	Subsequent sexing F–I	SM	MM
(A) All usable photographs (MQ ≥ 2; 74 individuals, 191 comparisons).				
F–I	Same year	99		
	Different year	11	4	
SM	Same year	1	21	
	Different year		3	
MM	Same year			41
	Different year			11
(B) High-quality photographs (MQ ≥ 3; 35 individuals, 56 comparisons).				
F–I	Same year	33		
	Different year	3		
SM	Same year		4	
	Different year			
MM	Same year			11
	Different year			5

Note: F–I, female – immature male; SM, subadult male; MM, mature male.

samples, and the results from the *SRY* method were in agreement with those from the *ZFY* method.

Sex determination of free-swimming individuals from genetic analysis of biopsy samples and melon-profile photographs was in agreement for 9 out of 10 animals (Table 1). One animal (No. 143) was genetically sexed as a male but was classified as female – immature male based on melon morphology.

Photographic sexing

We were able to assign an age–sex category (MQ ≥ 2) to 356 of the 1059 melon photographs. This accounted for 168 different fin identifications (110 female – immature males, 24 subadult males, and 34 mature males). Seventy-four individuals had more than two melon photographs (MQ ≥ 2), allowing 191 comparisons to be made, the majority of which spanned only a single field season (n = 162). Sequential melon photographs (MQ ≥ 2) almost always yielded the same age–sex category (Table 2A) and high quality melon photographs always yielded the same age–sex category (Table 2B). One individual (No. 31) was initially categorized as female – immature male in 1990 and subsequently categorized as a subadult male in 1994; however, high quality melon photographs taken in 1995 and 1996 indicate that this whale should be considered a female – immature male (Table 3). Another three individuals (Nos. 28, 102, and 267) were identified as female – immature male early in the study and subsequently identified as subadult male (Table 3). These individuals may have been males displaying the onset of sexual maturity, although the low quality of most of the melon photographs may also have influenced the results.

There were 30 sets of three sequential melon photographs of the same individual, each taken on separate days. In only one set was there an incongruity in categorization between photographs (individual No. 531; see Table 3). This is equivalent to an error rate of 3.3%. Thus, while interpretation of quality-2 melon photographs did result in a miscategorization, the error rate was very low.

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Table 3. Individuals that were assigned more than one age–sex class over the duration of the study.

	Age–sex categorization based on melon photographs taken in:					
ID No.	1990	1993	1994	1995	1996	1997
28	F–I (2)					SM (2)
102	F–I (2)	F–I (2)			SM (2)	SM (2)
267	F–I (2)	F–I (2)			SM (2)	SM (3)
531	F–I (2)		SM (2)	F–I (3)	F–I (4)	

Note: Values in parentheses are the highest melon photograph quality (1, unusable; 4, excellent). F–I, female – immature male; SM, subadult male.

Discussion

Reliable identification of sex is important in studies of free-living animals. For many species of cetaceans, this may be difficult to achieve from field observation alone. However, photographs of sexually dimorphic features have been shown to allow sex identification in some species (e.g., size–head shape for sperm whales, Gordon 1987; dorsal fin size and shape for killer whales (*Orcinus orca*), Olesiuk et al. 1990). Photographs of the melon profile are shown here to be reliable for determining the age–sex class of northern bottlenose whales. Molecular sexing (e.g., using biopsy samples) offers an alternate technique for sex identification when individuals cannot be differentiated by morphological means (e.g., females and immature males).

The *ZFY* and *SRY* methods of molecular sexing were shown to be reliable for northern bottlenose whales from the tests on known-sex samples and through cross-validation of these methods. In the one case where the results from molecular sexing (*SRY* method = female) were not in agreement with the documented sex (male), it is likely there was either inhibition at the *SRY* locus or an error in the whaling records. Based on the test results, we are confident that molecular identification of the sex of the 20 biopsied animals from the Gully was accurate.

We suggest that the *ZFY* and *SRY* methods be used in conjunction for molecular sexing, to overcome the potential limitations of each, especially in studies of taxonomic groups about which little is known biologically or genetically. As degraded samples may not allow amplification of the *ZFY–ZFX* fragment, primers targeting a smaller fragment of this locus may be required if this method is to be used. There remains some uncertainty about the exact position of the *Taq*I restriction sites in this fragment (Palsbøll et al. 1992). The *SRY* method is better suited to analysis of degraded samples and may be more reliable, as it targets a Y-specific locus (Sinclair et al. 1990) rather than relying on the conservation of sex-specific restriction sites on homologous loci found in both sexes. Ideally, *SRY* reactions should be multiplexed with another nuclear locus rather than with a mtDNA fragment, as used in this study, to avoid PCR competition favouring amplification of the latter (multiple copies per mitochondrion, multiple mitochondria per cell) at the cost of the *SRY* locus (single nuclear copy). If this is not possible, samples should first be tested for their ability to amplify a nuclear fragment similar in size to the *SRY* target (147 bp), to identify degraded samples that may give false negatives if a mitochondrial fragment is co-amplified with the *SRY* locus.

The overall agreement in sex identification from genetic analysis and melon-profile photographs for the 10 animals assayed using both methods (Table 1), and the low rate at which animals were miscategorized (3.3%), suggests that determining sex from melon morphology alone may be reliable for most age–sex classes in field studies of northern bottlenose whales. However, although females and immature males could not be immediately distinguished by melon morphology alone, a larger data set spanning a greater portion of an animal's life history (e.g., up to a decade or more) may allow reliable sex identification of all age–sex classes with hindsight (e.g., killer whales; Olesiuk et al. 1990).

Information obtained from this study may also help determine the age of onset of sexual maturity in males in the Gully. By collecting individual melon photographs over a sufficient period of time, males can be recognized as they develop the distinctive subadult and mature-male melon shape. None of the individuals that displayed a change in melon shape have been aged, although sighting histories can be used to set a minimum age. Age–length curves from whaling data (Christensen 1973) indicate that "adult-sized" whales are at least 2 years old. Each of the three whales that appeared to mature over the course of the study were adult-sized when they were first observed (Gowans 1999). The minimum age for these whales when they began to show a change in melon shape ranged from 8 to 10 years and this fits well with the whaling-data estimates for the onset of puberty (5–12 years; Christensen 1973; Benjaminsen and Christensen 1979). However, these age estimates represent only a minimum estimate, not an actual age, and as the initial categorizations were based only on low-quality photographs (MQ of 2), it is difficult to distinguish between the effect of photograph quality and a change in melon shape in these cases. The individual that was genetically sexed as male and identified as female – immature male by melon morphology (No. 143) would have been at least 9 years old when the last melon photograph was taken in 1996, indicating that the development of the male-shaped melon may occur some time after 9 years of age (Gowans 1999). Until it is possible to determine at what age the melon profile begins to change shape in males, it will not be possible to classify individuals as adult females, despite their long sighting history with a female – immature male melon profile.

In conclusion, we think that melon morphology can reliably distinguish age–sex classes of northern bottlenose whales. This method could be adapted for other species that display gradual morphological changes during sexual maturation.

Acknowledgements

We thank those who collected field data during this study. We especially thank A. Faucher, who established the photographic-identification catalogue and initially investigated the possibility of using melon photographs to determine sex. Norwegian whaling samples were provided by I. Christensen, Institute of Marine Research, Norway. Canadian stranding samples were provided by the Nova Scotia Stranding Network, Halifax, N.S.; Groupe de recherche et education sur le milieu marine, Quebec; and the Department of Fisheries and

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Oceans Canada, Mont Joli, Quebec. Funding was provided by the Canadian Federation of Humane Societies, the Natural Sciences and Engineering Research Council of Canada (NSERC), the Whale and Dolphin Conservation Society, and World Wildlife Fund Canada. S.G. was supported by graduate scholarships from NSERC and the Izaak Walton Killam Memorial Scholarship; M.L.D. by a New Zealand Vice-Chancellor's Committee William Georgetti Scholarship and a University of Auckland Doctoral Scholarship; and S.K.H. by a Canadian Commonwealth Scholarship. We thank C.S. Baker and F.B. Pichler for their valuable suggestions regarding molecular-sexing protocols. J. Arch, R.W. Baird, J. Christal, A. Ottensmeyer, N. Patenaude, and three anonymous reviewers provided useful suggestions regarding the manuscript.

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